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Inhibitory effect of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene on 3T3-L1 adiopocyte differentiation

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Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytoalexin found in diverse plant species, including grapes and peanuts. The antioxidant, anticancer, and cardioprotective properties of resveratrol have been well-characterized. The anti-obesity effect of resveratrol has also been demonstrated in previous studies. In this study, we evaluated the effects of a resveratrol analogue, *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene, on adipocyte differentiation using 3T3-L1 cells. According to our results, the tested analogue potently inhibits the differentiation of 3T3-L1 adipocyte to a greater degree than resveratrol. Moreover, *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene strongly downregulated the expression of fatty acid metabolism-related proteins such as fatty acid synthase and acetyl-CoA carboxylase. These results point to the potential of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene as an obesity prevention agent.

1. Introduction

The prevalence of obesity is reaching worldwide epidemic proportions, and is currently increasing (James et al. 2004). Obesity is associated with increased risks of stroke, heart disease, and premature death (Strazzullo et al. 2010; Horwich and Fonarow 2010; Adams et al. 2006). Because fatty acid is an important component of lipids, fatty acid synthase (FAS) is generally regarded as a potential therapeutic target for obesity. The differentiation of preadipocytes and the induction of metabolic pathways associated with lipid metabolism induces FAS expression (Kim et al. 1998). The synthesis of fatty acids by FAS requires malonyl-CoA, the substrate for FAS and a regulator of fatty acid oxidation. Acetyl-CoA carboxylase (ACC) catalyzes the production of malonyl-CoA via the carboxylation of acetyl-CoA (Wakil and Abu-Elheiga 2009; Kreuz et al. 2009; Tong 2005). This enzyme performs a crucial function in fatty acid metabolism in animals, including humans.

Resveratrol (Fig. 1) is a natural *trans*-stilbenoid and exhibits various biological properties such as antioxidant, anticancer, and cardioprotective effects (Frémont 2000). Moreover, a number of previous studies have shown that resveratrol exerts anti-obesity effects (Szkudelska and Szkudelski 2010; Rivera et al. 2009; Ahn et al. 2007). In experiments on mice fed on a high-calorie diet, resveratrol improves the physiology of middle-aged mice and significantly increases their survival (Baur et al. 2006). Resveratrol also inhibits FAS and adipogenesis in adipocytes (Tian 2006; Rayalam et al. 2008). Resveratrol's anti-obesity effects involve the potent ability of resveratrol to reduce FAS synthesis and to inhibit ACC activity (Gnoni and Paglialonga 2009).

As a component of a study of resveratrol-related anti-obesity agents, we evaluated the effects of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene (Fig. 1) on adipocyte differentiation and fatty acid metabolism-related protein expression and compared it with the activity of resveratrol. *(E)*-1,2-Di(3,5-dimethoxyphenyl)ethene is a synthetic *trans*-stilbenoid (Kim et al. 2002) which resembles the structure of resveratrol but contains methoxy groups instead of the hydroxyl groups of the natural product. Our methods of examination involved the

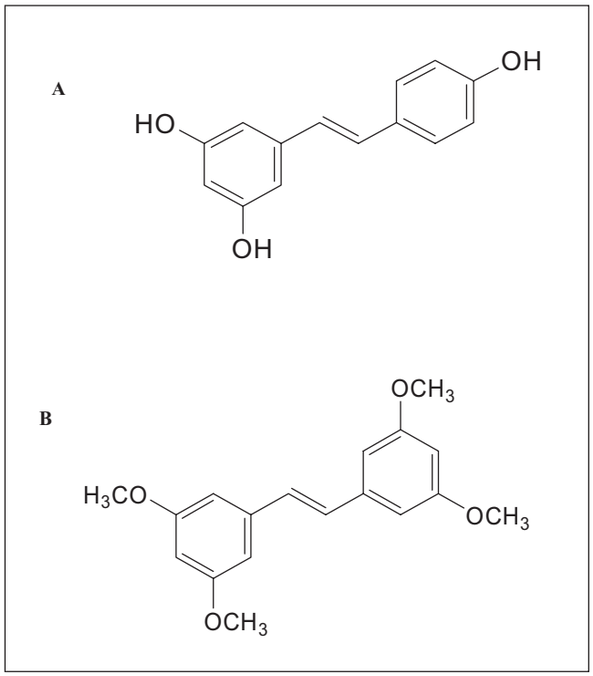

Fig. 1: (A) Structures of resveratrol and (B) *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene

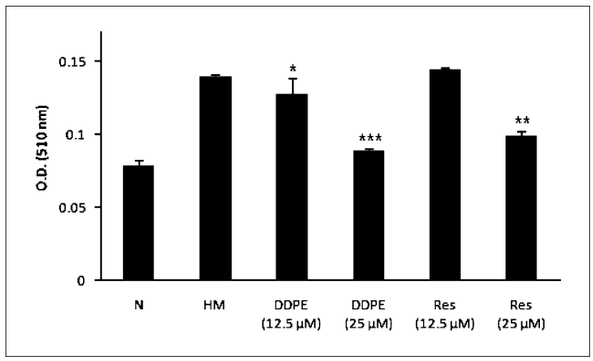

Fig. 2: Inhibitory effects on 3T3-L1 adipocyte differentiation
N: Normal, HM: Hormone mixture, DDPE: *(E)*-1,2-Di(3,5-dimethoxyphenyl)ethene, Res: Resveratrol. Adipocyte differentiation was measured by Oil Red O staining. Three independent experiments were conducted and the results were expressed as means ± S.D of absorbance at 510 nm

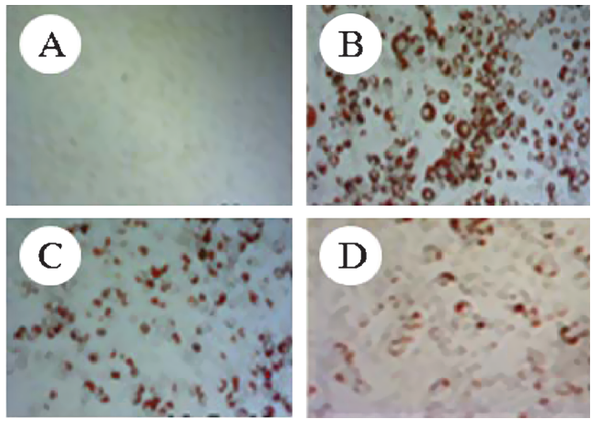

Fig. 3: Photographs of 3T3-L1 adipocytes after Oil Red O staining
A: Normal, **B**: Hormone mixture (including insulin, dexamethasone, and IBMX) treatment, **C**: Hormone mixture and 12.5 μM of DDPE treatment **D**: Hormone mixture and 25 μM of DDPE treatment

effects on hormone-induced cell differentiation and the expression of FAS and ACC in 3T3-L1 cells.

2. Investigations, results and discussion

2.1. Inhibitory effects on adipocyte differentiation

In order to determine whether *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene exerts an anti-obesity effect, its effects on adipocyte differentiation were evaluated using 3T3-L1 adipocyte. As is shown in Fig. 2, *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene significantly inhibited hormonal cocktail-induced adipocyte differentiation in a dose-dependent fashion as measured via Oil Red O staining. The inhibitory effect of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene was significantly higher than that of resveratrol at the same dose. The morphological changes of 3T3-L1 adipocyte are presented in Fig. 3. At 25 μM of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene, differentiation was almost suppressed.

2.2. Effects on the expression of proteins involved in fatty acid metabolism

It has been previously reported that fatty acid metabolism-related proteins such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) are crucial targets for the prevention of obesity. Thus, we subsequently attempted to determine whether *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene can modulate the expression of FAS and ACC in 3T3-L1 adipocytes. After adipocyte differentiation was completed in the presence or absence *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene, protein expressions were evaluated via Western blot analysis. As shown in Fig. 4, hormonal cocktail-induced FAS and ACC expressions were inhibited significantly by 25 μM of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene, to a greater degree than was achieved with resveratrol. These results suggest that *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene inhibits adipocyte differentiation via the suppression of fatty acid metabolism-related expressions of proteins such as FAS and ACC; therefore *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene may prove useful for the prevention of obesity.

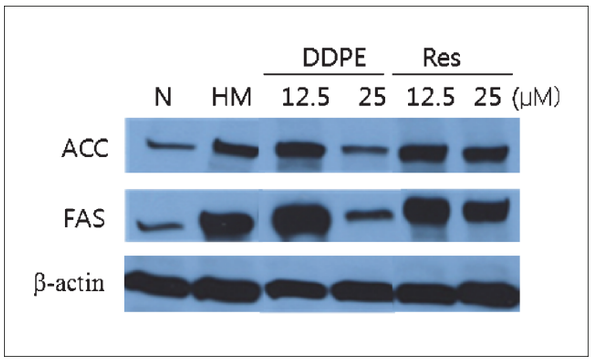

Fig. 4: Effect on intracellular expression of ACC and FAS
N: Normal, HM: Hormone mixture, DDPE: *(E)*-1,2-Di(3,5-dimethoxyphenyl)ethene, Res: Resveratrol

In conclusion, this study is the first to demonstrate that *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene, a resveratrol derivative, potently inhibits adipocyte differentiation via the FAS and ACC downregulation. Although it remains unknown whether *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene will have anti-obesity effects in animals and human, we present potent inhibitory effects on hormonal cocktail-induced adipocyte differentiation and FAS and ACC expressions in this study. Our future study will investigate its anti-obesity effect and safety in animal models.

3. Experimental

3.1. Instrumentations and materials

The ^{1}H-(300 MHz) and ^{13}C-(75 MHz) NMR spectra were run on a Gemini-2000 spectrometer. The FABMS spectra were measured on a Hewlett-Packard mass spectrometer. The UV spectra were determined using a Molecular Devices E09090 microplate reader. 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). Resveratrol, Oil Red O, insulin, 1-methyl-3-isobutyl xanthine (IBMX), and dexamethasone were purchased from Sigma (St Louis, MO). Antibodies against fatty acid synthase and acetyl CoA carboxylase were purchased from Cell Signaling Technology (Beverly, MA).

3.2. Synthesis of (E)-1,2-di(3,5-dimethoxyphenyl)ethene

The synthesis of *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene was conducted using the previously reported method (Kim et al. 2002) with some minor modifications. After dissolving 3,5-dimethoxybenzyl bromide (3 g, 13.2 mmol) in anhydrous benzene (44 ml), triphenylphosphine (8.38 g, 19.8 mmol) was added and refluxed for 2 h. A precipitate was generated after cooling. 6.58 g (99%) of 3,5-dimethoxybenzyl triphenylphosphonium bromide was obtained via filtration. In 12.3 ml of methylene chloride, 3,5-dimethoxybenzyl triphenylphosphonium bromide (150 mg, 0.3 mmol), 3,5-dimethoxy benzaldehyde (37.1 mg, 0.2 mmol), and tetrabutylammonium bromide (8 mg) were dissolved. Then, a 50% sodium hydroxide aqueous solution (1.1 ml) was gradually introduced to the above solution. After 5 h of stirring, the reaction mixture was poured into water

and extracted with CH_2Cl_2. The extract was washed in brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified via flash column chromatography on a silica gel (eluent: CH_2Cl_2), to give 41.2 mg of 1,2-di(3,5-dimethoxyphenyl)ethene as a mixture of *E* and *Z* isomers. To the solution of this mixture in heptanes (5 ml) was added a catalytic quantity of iodine and then heated to reflux for 12 h. The reaction mixture was cooled to room temperature and the generated solid was obtained via filtration to give the desired product, *(E)*-1,2-di(3,5-dimethoxyphenyl)ethene (29.3 mg).

(E)-1,2-Di(3,5-dimethoxyphenyl)ethene. White solid; ^{1}HNMR ($CDCl_3$, 300 MHz) δ: 6.94 (2H, s), 6.60 (4H, d, J = 2.4), 6.33 (2H, t, J = 2.4), 3.77 (12H, s), 7.00 (1H, t), 6.97 (1H, t), 6.79 (1H, t), 6.76 (2H, m), 3.88 (3H, s), 3.84 (3H, s). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 160.99, 139.16, 129.2, 104.65, 100.14, 55.38. MS (EI^+) m/z 300(M^+, 100), 269(40), 254(22), 238(15), 211(12).

3.3. Cell culture and differentiation

3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum at 37 °C in a 5 % CO_2 incubator. 3T3-L1 cells were maintained in 48-well culture plates. After 100% confluence was reached, the cells were treated for 48 h with a hormone mixture containing 10 μg/mL insulin, 0.5 μM dexamethasone, and 0.5 mM IBMX, and then exchanged with DMEM containing insulin. The cells were differentiated into adipocytes until day eight in the presence or absence of stimuli.

After finishing adipocyte differentiation, the TG contents were evaluated via Oil Red O staining. The cells were washed twice in phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS. Adipocytes were incubated for 1 h with Oil Red O dye, which stained as red. The lipid droplets were dissolved in isopropanol and measured at 510 nm.

3.4. Western blotting

The cells were washed twice in ice-cold PBS after stimulation and lysed in lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, and 0.2% protease inhibitor cocktail, pH 7.2). Proteins were separated via SDS–PAGE. Western blot analysis was conducted with specific antibodies.

3.5. Statistical analysis

The data are expressed as the means ± SD from three independent experiments. Statistical analysis was conducted via one-way ANOVA followed by Tukey's test.

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